Studies on the Biologically Active Substances of Sapium japonicum (Euphorbiaceae) (Commemoration Issue Dedicated to Professor Minoru Ohno On the Occasion of his Retirement)

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Studies on the Biologically Active Substances of *Sapium japonicum* (Euphorbiaceae)

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A piscicidal constituent and an antifungal constituent were isolated from *Sapium japonicum*. The structure of the piscicidal constituent, $C_{32}H_{42}O_8$, was shown to be 12-*O*-*n*-deca-2,4,6-trienoyl-phorbol-(13)-acetate, and the structure of the antifungal constituent was shown to be methyl 8-hydroxy-5,6-octadienoate.

I. INTRODUCTION

From the beginning of man we have utilizing several kinds of plants as medicines, insecticides, insect repellents, antiseptics, fish poisons and so on. With the progress of chemistry, the biologically active principles of plants have been revealed and new remedies, pesticides have been developed referring to them. And further studies on various kinds of active substances in plants are being planned and results of great significance are expected.

Up to date extensive chemical studies have been carried out mainly on medicinal plants and insecticidal plants.

From medicinal plants a variety of cardiac glycosides and alkaloids have been isolated and their structures have been established. Recently more than 10,000 plant extracts were screened for antitumor activities against the Leukemia L-12120 in mice and the Walker 256 carcinosarcoma *etc.* by two groups of scientists in the U.S.A.[1] Among the plants tested effective plant species are *Stephania hernandiifolia, Elephantopus elatus, Acnistus arborscens, Catharanthus roseus* and *Acronychia baueri,* from which (±)- tetrandrine, elephantopin and elephantin, withaferin A, vincaleukoblastine, and acronychin were isolated as the active constituents, respectively.

On the other hand several compounds which have insecticidal activities were isolated from plants. Rotenoids are typical examples among them. Subsequently mammeins, quassin and ryanodine were found to be active constituents of *Mammea americana, Quassia amara* and *Tripterygium wilfordii,* respectively.

It should be emphasized that most plants possessing such biological activities as mentioned above are also known to be poisonous for fish. Plants possessing piscicidal activity are therefore available for some kinds of medicines or insecticides.

The piscicidal plants are mainly found in the tropics or the subtropics where they have been employed for catching fish by natives. Those known as piscicidal plants are listed up in Table I-1.[2,3] The piscicidal activity has been found in numerous plants belonging particularly to the family Leguminosae or Euphorbiaceae. Especially in

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the last five years, plants in Euphorbiaceae have been chemically investigated successfully because of their attractive biological activities.[4~9]

Table I–1. Piscicidal Plants*

Myricaceae	*Myrica sapida*	bark
Juglandaceae	*Juglans mandshurica*	root, fruits
Polygonaceae	*Polygonum orientale*	leaves
Menispermaceae	*Anamirta paniculata*	fruits
	Cocculus indicus	root
	Cissampelos pareira	root
	Stephania hernandifolia	root
Theaceae	*Camelia drupifera*	
	Ternstroemia sp.	bark
	Schimaliukiuensis	bark
Guttiferae	*Calophyllum inophyllum*	leaves, fruits
	C. muscigerum	bark
Capparidaceae	*Gynandropsis gynandra*	seeds
Pittosporaceae	*Pittosporum ferrugineum*	leaves, fruits
Leguminosae	*Acacia catechu*	root
	Albizzia acle	bark
	A. saponaria	bark
	A. procera	bark
	Entada phaseoloides	fruits
	Derris elliptica	root
	D. montana	root
	D. pubipetala	root
	D. scandens	latex
	D. robusta	
	Lonchocarpus sericeus	
	Milletia ichthyochtona	seeds
	M. sericea	root
	M. taiwaniana	fruits
	Pachyrrhizus erosus	seeds
	Pithecellobium ellipticum	bark
	Pongamia pinnata	seeds, root
	Robinia pseud-acasia	
	Tephrosia candida	root, leaves
	T. toxicaria	bark
	T. vogelii	
Erythroxylaceae	*Erythroxylon cuneatum*	
Euphorbiaceae	*Alchornea parviflora*	whole plant
	Cleistanthus collinus	leaves
	Excoecaria agallocha	latex
	E. cochinchinensis	latex
	Euphorbia antiquorum	latex
	E. neriifolia	leaves
	E. tirucalli	latex
	E. trigona	latex
	Hura crepitans	latex
	Jatropha curcas	latex
	Mallotus apelta	seeds
	M. philippinensis	latex
	Sapium indicum	fruits

(continued on next page)

Table I–1. (continued from the preceding page)

Rutaceae	*Acronychia resinosa*	bark
	A. laurifolia	root
	A. odorata	bark
	Zanthoxylum piperitum	bark, fruits
Meliaceae	*Dysoxylon arborescens*	
	Melia azedarach	
	Walsura piscidia	bark
Sapindaceae	*Sapindus saponaria*	seeds, fruits
	Serjania sp.	
Buxaceae	*Buxus rolfei*	fruits
Tiliaceae	*Grewia* sp.	
Sterculiaceae	*Pterospermum diversifolium*	root
Thymelaeaceae	*Wiksroemia ridleyi*	bark
Flacourtiaceae	*Casearia graveolens*	fruits
	C. tomentosa	fruits
Lecythidaceae	*Barringtonia* sp.	seeds, bark
Umbelliferae	*Hydrocotyle javanica*	leaves
Primulaceae	*Anagalis arvensis*	whole plant
Myrsinaceae	*Maesa cumingii*	leaves
	M. pyrifolia	bark
	Aegiceras corniculatum	bark
Ebenaceae	*Diospyros ebenaster*	immature fruits
	D. lucida	fruits
	D. wallichii	fruits
Styracaceae	*Styrax japonica*	fruits
Apocynaceae	*Thevetia peruviana*	wood
Rubiaceae	*Gardenia currani*	fruits
	Randia dumetorum	immature fruits
Verbenaceae	*Callicarpa candicans*	leaves
	Vitex trifolia	bark, fruits, leaves
Acanthaceae	*Jasticia hayatai* var. *decumbens*	whole plant
Caprifoliaceae	*Viburnum awabuki*	leaves
Compositae	*Ichthyothere terminalis*	leaves
	Spilanthes acmella	whole plant
Dioscoreaceae	*Dioscorea tokoro*	root
Palmae	*Rhapis cochinchinensis*	fruits

* Presented at the 6th conference on the chemistry of natural products, July 23, 1971, Chino, Nagano Pref.

The active principles already isolated from piscicidal plants are classified as follows: rotenoids, coumarins, lignans, terpenoids, polyacetylenes, alkaloids, saponins, toxalubumins. Some of piscicidal constituents isolated are summarized in Table I-2.

Although investigations on chemical constituents of the piscicidal plants have not been carried out as extensively as those of medicinal plants or insecticidal plants, it is interesting and important to investigate chemical constituents of the piscicidal plants in aiding the discovery of new compounds having any biological activity.

The following chapters deal with the isolation and the structural elucidation of a piscicidal and an antifungal constituent of *Sapium japonicum* together with their biological activities.

Table I–2. Piscicidal Constituents and their Sources

Compound		Source
Rotenoids[10]	rotenone, deguelin, elliptone	*Derris elliptica*
	tephrosin, toxicarol	*Tephrosia* species
	munduserone[11], pachyrrizone[12]	*Mundulea sericea* & *Pachyrrhizus erosus*
Coumarins	erosnin[13], pachyrrizin[12]	*Pachyrrhizus erosus*
	mammein[14],	*Mammea americana*
	(+)-inophyllolide[15] and its derivatives	*Calophyllum inophyllum*
Lignans	diphyllin, collinusin, cleistanthin	*Cleistanthus collinus*[16,17] & *Justicia procumbens*[18]
	justicidin A, justicidin B	*Justicia hayatai* var. *decumbens*[19]
	justicidin A, B, C, D, E, F and diphillin	*Justicia procumbens*[20,21]
Terpenoids	picrotoxinin, picrotin	*Anamirta paniculata*[22]
	callicarpone[23]	*Callicarpa candicans*
	maingayic acid[24]	*Callicarpa maingayi*
	huratoxin[25]	*Hura crepitans*
Polyacetylenes	ichthyothereol and its acetate[26]	*Ichthyothere terminalis*
Alkaloids	bebeerine, sepeerine, cissampareine and 4″-0-methylcurine	*Cissampelos pareira*[2,3]
	isotrilobine, (±)-tetrandrine, fangchinoline, isochondrodendrine	*Stephania hernandiifolia*[2,3]
Saponins[1,2]		

II. BIOLOGICAL ACTIVITIES OF *SAPIUM JAPONICUM*

At present a few kind of piscicidal plants are found in Japan, because they are no longer used for catching fish. If the plants belonging to specified families, such as Euphorbiaceae, are screened for piscicidal activity, more plants which are poisonous for fish may be discovered in Japan.

Sapium japonicum Pax *et* Hoffm. (Euphorbiaceae) is a deciduous high tree commonly found in the bases of mountains of Japan. Plants belonging to the family Euphorbiaceae are distributed in the tropics, subtropics and temperate zone and are classified into about 280 genera, 8000 species. The family Euphorbiaceae contains many species which have been used as folk remedies and also contains many species of the poisonous variety. Some of them in this family contain milky sap to which their toxicity is attributed.

Chemists have always shown a great interest in the poisonous plants in Euphorbiaceae. *Croton tiglium,* originated in south-east Asia, is a small tree whose seed oil (Croton oil) is poisonous. The oil, however, has been used as a purgative by the natives. It had also been known to be poisonous against fish or amphibia, and to inflame the skin. Although chemical investigations to isolate the toxic substances in this oil had been carried out,[27] the active principles had not been isolated till quite recently, and only several fatty acids had been revealed as the constituents of this oil.[28] Recently, several compounds with tumor promoting and inflammatory activities have

been isolated as the toxic components successfully.[4~9, 29~33]

Although the natural beauty of *S. japonicum* is well known by everyone, the chemical constituent of the biological active component have received little attention.[34] However, since *S. japonicum* slightly exudes milky sap when the young twigs are broken off, the existence of some toxic substance was expected. The bioassay on *Oryzias laptipes* (killie-fish, himedaka in Japanese) concerning the methanol extract of the leaves confirmed the occurrence of some piscicidal constituent.

It was worthy of our notice that no diseased spot could be found on its leaves. This aspect suggested the existence of some antifungal constituent in this plant. This assumption was confirmed by conidia germination test using *Cochliobolus miyabeanus*.

On the basis of these observations the necessity for a re-newed interest in the biological activities of *S. japonicum* is received. Hence, the studies to reveal the active principles were started.

III. STUDIES ON A PISCICIDAL CONSTITUENT OF *SAPIUM JAPONICUM*

III-1. Extraction and Isolation of a Piscicidal Constituent of *Sapicum japonicum*

The extraction and isolation were controlled by killie-fish bioassay.

It was expected that the poisonous activity of *S. japonicum* was attributed to the milky sap slightly exuded from its barks or twigs. Preliminary experiments, seeking which part of this plant was the most effective for the isolation, were carried out, and the results are summarized in Table III-1. Each methanol extract of leaves, twigs, and

Table III-1. Piscicidal Activity of Leaves, Twigs and Barks of *S. japonicum*.

	leaves		twigs		barks	
	yield, mg	activity ppm	yield, mg	activity ppm	yield, mg	activity ppm
hexane-soluble fr.	6.8	10.0	4.3	5.0	11.4	2.5
benzene-soluble fr.	2.2	10.0	1.0	2.5	0.8	1.5
EtOAc	35.2	—	6.6	—	8.0	—

One gram of each part was extracted with methanol. The methanol extracts were concentrated and the aqueous concentrates were extracted with *n*-hexane, benzene and ethyl acetate successively. Each fraction obtained thus was tested for the piscicidal activity.

barks was extracted with *n*-hexane, benzene and ethyl acetate successively. The piscicidal activity was found in both of the *n*-hexane and benzene extracts, and the extracts obtained from barks showed the most prominent activity and those from twigs followed. Therefore the young twigs and barks in June and July were collected.

The benzene soluble fraction of the methanol extracts of the young twigs and barks was chromatographed on granular charcoal and eluted with water containing an increasing ratio of acetone. The activity was found in the fraction eluted with 100% acetone. Purifying this fraction by adsorption column chromatographies of Florisil, silicic acid-Celite 545 and charcoal successively as shown in Fig. III-1, a

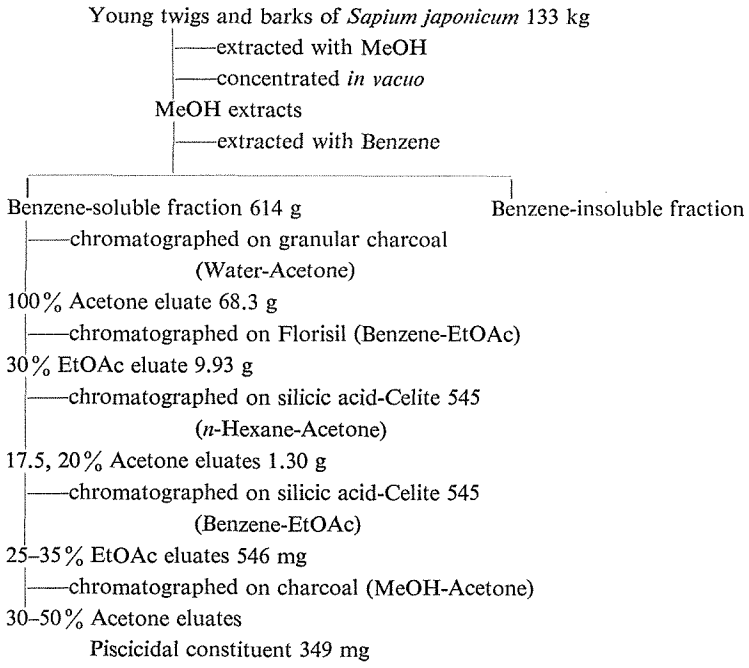


Fig. III–1. Extraction and Isolation Procedure.

piscicidal constituent (**I**) was isolated as a colorless glassy resin in a total yield of 349 mg from 133 kg of the young twigs and barks.

The compound (**I**) was able to be detected as a dark shadow spot under uv lamp on a thin layer plate of silica gel GF_{254} developed with a solvent (benzene-acetone 8:3, *Rf* 0.5, *n*-hexane-acetone 6:4, *Rf* 0.4). It was visualized by characteristic reddish brown coloration on spraying with 5% vanillin in conc. sulfuric acid, and after a few minutes it turned violet and blue on heating.

The compound (**I**) is soluble in chloroform, benzene, ethyl acetate, ether and methyl alcohol, but not in water. It decomposes itself rapidly under drying up in the air.

The piscicidal constituent (**I**), $[\alpha]_D^{28.5}$ $-19.6°$ (*c* 0.88, CH_3OH) had the uv absorption (EtOH) at 232 sh. (ε 9.24×10^3), 307 nm (ε 3.59×10^4) and the ir spectrum ($CHCl_3$) (Fig. III-2) shows the absorption bands at 3200—3500 (broad), 1740 (sh.), 1725, 1710, 1620 and 1260 cm^{-1}. The mass spectrum of **I** is reproduced in Fig. III-3, and exhibits the molecular ion peak at *m/e* 554. The pmr spectrum (90 MHz, $CDCl_3$)* shows numerous signals at a region of 0.5—8.0 ppm as given in Fig. III-4.

The uv absorption maximum at 307 nm together with the ir absorption band at 1620 cm^{-1} suggested the presence of a long conjugated system in **I**. This was also supported by the appearance of signals at a region of 5.5—7.5 ppm in the pmr spectrum.

The broad and intense ir absorption band at 3200—3500 cm^{-1} indicated the

* In this chapter unless otherwise stated, the pmr spectra were taken in deuteriochloroform at 90 MHz and chemical shifts were expressed as δ values (ppm) from tetramethylsilane as internal standard and coupling constant in Hz. Singlet, doublet, triplet, quartet, double doublet and multiplet are abbreviated to s., d., t., q., d.d., and m., respectively.

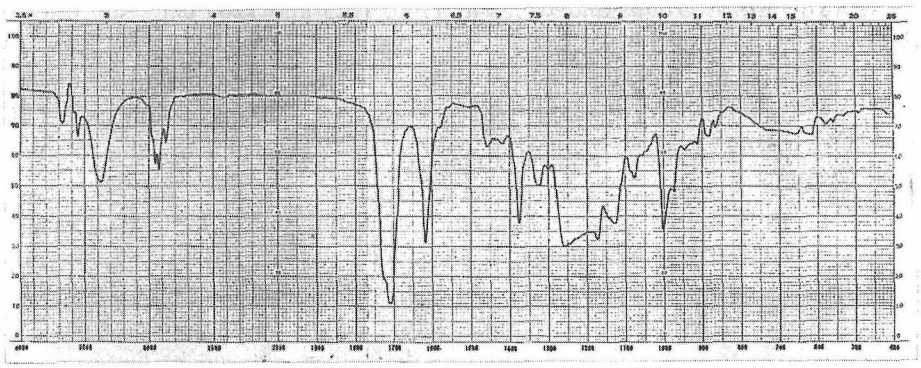


Fig. III–2. The ir spectrum of **I** ($CHCl_3$).

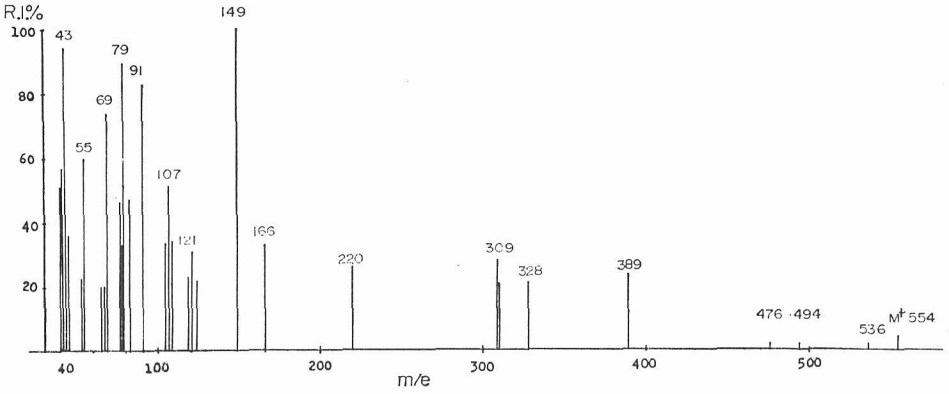


Fig. III–3. The mass spectrum of **I**.

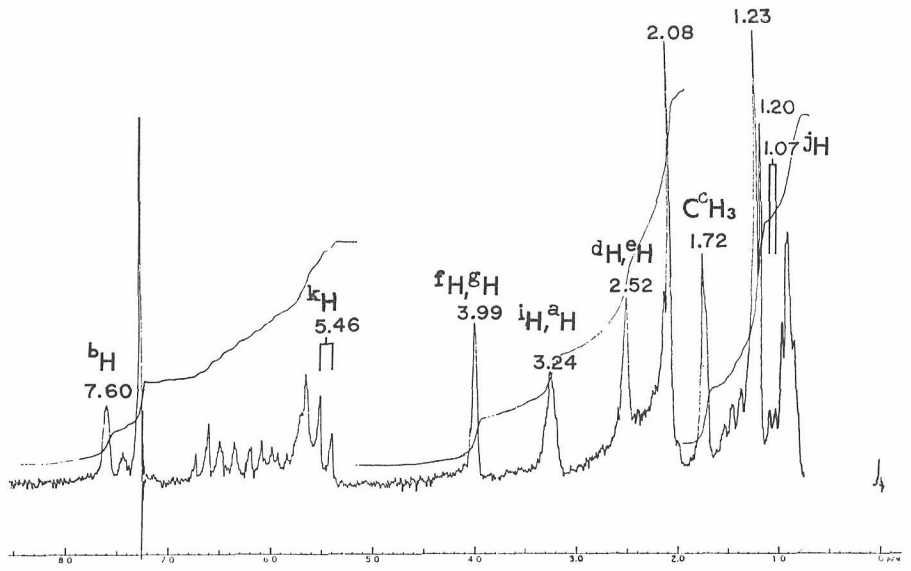


Fig. III–4. The pmr spectrum of **I** (90 MHz, $CDCl_3$).

presence of hydroxyl groups and bands at 1740, 1725 and 1260 cm^{-1} suggested that ester groups are contained in **I**.

Its pmr spectrum indicated the presence of six methyl groups attributable to a primary methyl (δ 0.89, t., J=6), a secondary methyl (0.87, d., J=6), two tertiary methyls (1.20 and 1.23, s.), a vinyl methyl (1.72, broad s.) and a methyl due to an acetyl group (2.08, s.).

In order to grasp the pmr spectral data, each protons, which are important for the structure argument of **I**, were tentatively named ^{a}H, ^{b}H, ^{c}H, ... as depicted in Fig. III-4.

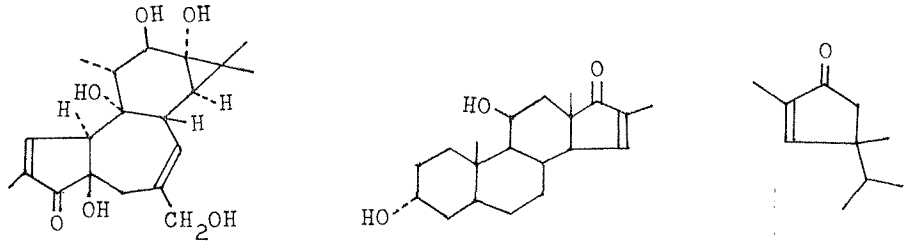
The chemical structure of **I** were estimated by the detailed discussions of the chemical and spectroscopic data of **I** and its derivatives. It will be discussed in the following section.

III-2. Chemical Structure of the Piscicidal Constituent (I) of *Sapium japonicum*

A. Partial structures of **I**

i) The presence of an α-methyl-α,β-unsaturated cyclopentenone system

The characteristic uv absorption at 232 nm and the ir absorption band at 1710 cm^{-1} were in good agreement with absorptions due to α-methyl-α,β-unsaturated cyclopentenone system as investigated in the compounds illustrated in Fig. III-5.[7,35,36] The presence of the system was also supported by the pmr spectrum. In the pmr spectrum of **I** one-proton broad singlet (^{b}H) at 7.60 ppm and three-proton broad singlet ($C^{c}H_3$) at 1.72 ppm were attributed to the β-proton and α-methyl protons of this system respectively.[37]



7) 35) 36)

1698 cm^{-1} 1701 cm^{-1} 1707 cm^{-1}

235 nm(ε 5200) 243 nm(ε 6850) 230 nm

Fig. III-5. Spectroscopic data of α-methyl-α,β-unsaturated cyclopentenone system.

The spin-decoupling experiments explained clearly not only the relationship between ^{b}H and $C^{c}H_3$ but also the presence of a proton (^{a}H) at γ-position of this enone system. One-proton broad singlet at 7.60 ppm collapsed to a doublet on double irradiation at the frequency of the methyl protons on the double bond ($C^{c}H_3$), and on the reverse procedure collapsed the broad singlet at 1.72 ppm to a doublet, as shown in Fig. III-6. Newly observed coupling constants (J_{ab}=2.0, J_{ac}=2.5) of the doublets

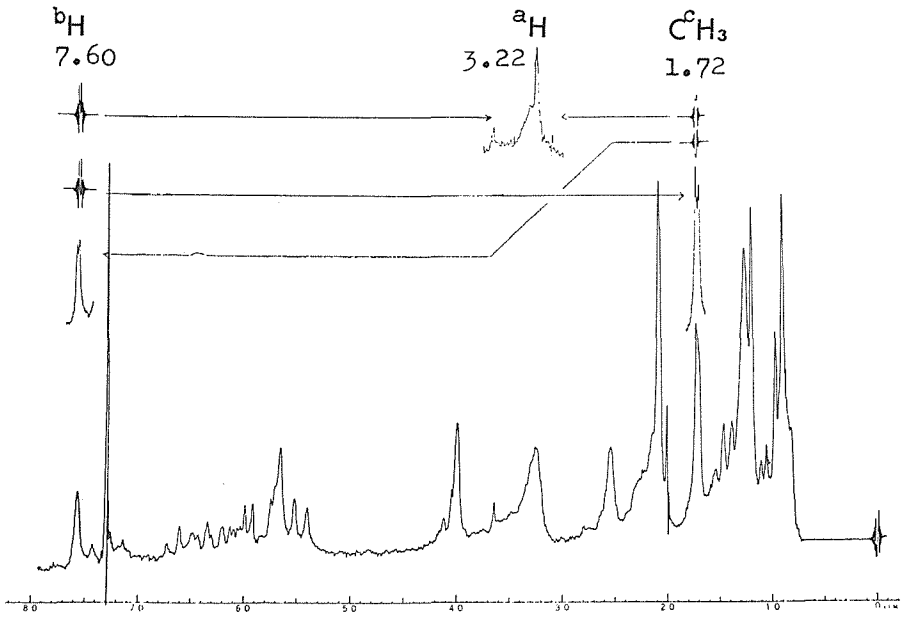
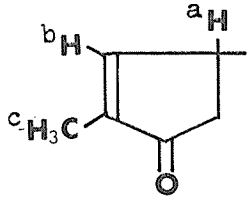


Fig. III–6. Spin-decoupling experiments-1.

at 1.72 and 7.60 ppm expressed the existence of a proton (^{a}H) at γ-position of this enone system. A one-proton multiplet (^{a}H) at 3.22 ppm was reduced to a singlet by irradiating at both the frequency ^{b}H and $C^{c}H_3$ simultaneously as shown in Fig. III-6. This procedure also indicated that no more hydrogen exists on this ring.

Thus the partial structure (i) is presented.



(i)

ii) Characterization of hydroxyl groups

In the pmr spectrum of **I** in $CDCl_3$, assignments of hydroxyl protons were unsuccessful even with D_2O treatment, because signals due to hydroxyl protons appeared as broad signals overlapping with other signals. However, the pmr spectrum of **I** in d_6-acetone gave three signals due to hydroxyl protons at 5.26, 4.72 and 3.7~3.9 ppm which disappeared after shaking with D_2O.

On acetylation with pyridine-acetic anhydride, **I** gave a monoacetate (**II**). In the pmr spectrum of **II** (Fig. III-7) a methyl signal due to a newly formed acetyl group appeared at 2.03 ppm, and the two-proton singlet at 3.99 ppm of **I** was shifted downfield by 0.49 ppm to 4.48 ppm. These observations indicated that one hydroxyl group among three is primary and the remaining two are tertiary. The two protons (^{f}H

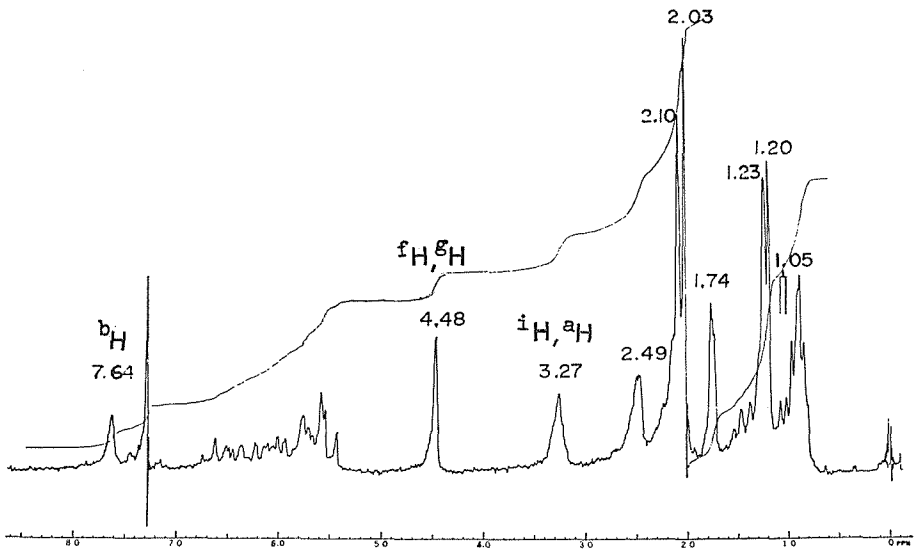
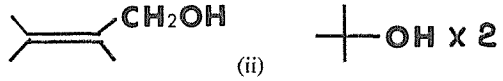


Fig. III–7. The pmr spectrum of monoacetate (**II**) (90 MHz, $CDCl_3$).

and ^{g}H) due to hydroxymethyl group resonated at a lower region (3.99 ppm) than usual. This fact suggested that the hydroxymethyl group is attached to a double bond.

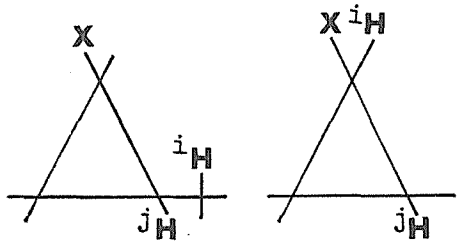
Here partial structures (ii) are proposed.



(ii)

iii) The presence of a cyclopropane ring

In the pmr spectrum of **I**, a one-proton doublet (^{j}H) which occurred at abnormally high field (1.07 ppm) was expected to be a signal corresponding to a proton attached to a cyclopropane ring which possessed an electron withdrawing group (X).[38] The relationship between the proton (^{j}H) and the group (X) was required to be vicinal on the ring.[38] As shown in Fig. III-8 the doublet (^{j}H) collapsed to a singlet on irradiation at the frequency of a proton (^{i}H) at 3.30 ppm, though the reverse experiment was unsuccessful because multiplet due to ^{a}H overlapped with that of ^{i}H. If the proton (^{i}H) is placed on the cyclopropane ring, the proton (^{i}H) should be bonded to the carbon atom bearing the group (X). Then the relationship between ^{j}H and ^{i}H was confirmed as partial structure (iiia) or (iiib).



(iiia) (iiib)

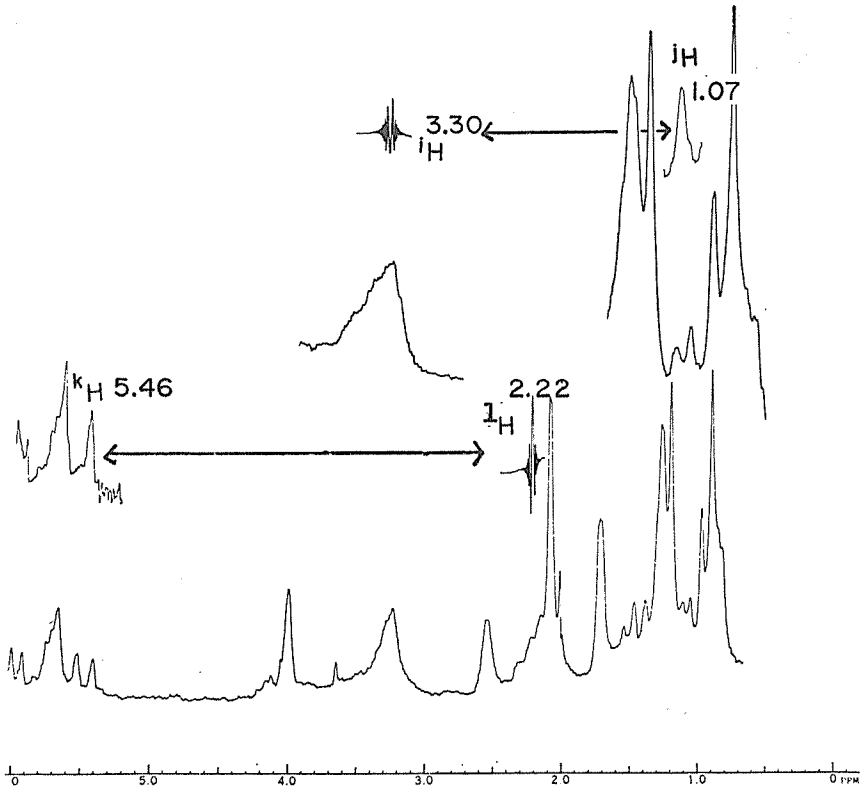


Fig. III–8. Spin-decoupling experiments-2.

iv) Characterization of a secondary methyl group by spin-decoupling experiments

Spin-decoupling experiments of **I** in a solvent ($CDCl_3$: C_6D_6 2:1) confirmed that protons (C^mH_3) due to a secondary methyl group at 0.90 ppm was coupled to a proton (^1H) resonated at 2.16 ppm. As shown in Fig. III-9, the multiplet at 2.16 ppm reduced

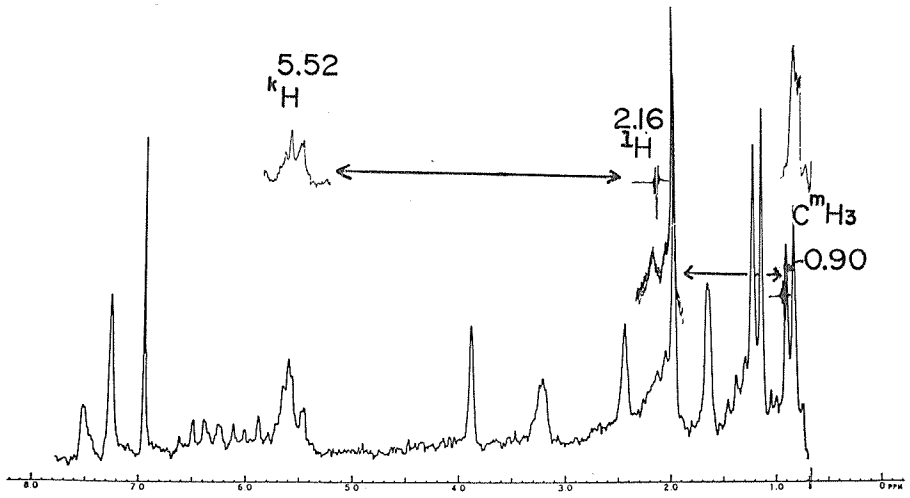
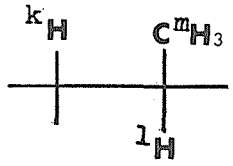


Fig. III–9. Spin-decoupling experiments-3 ($CDCl_3$:C_6D_6 2:1).

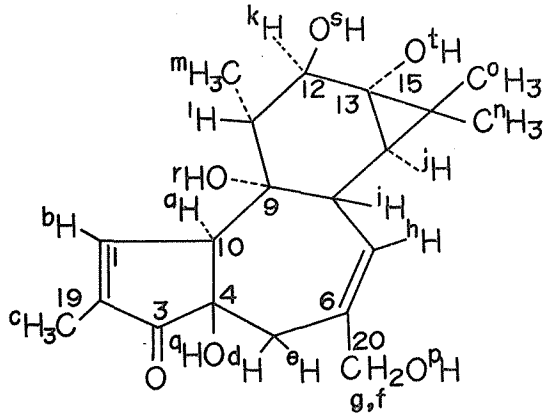
to a doublet on irradiation at the frequency of the methyl protons. On the other hand, a one-proton doublet (^{k}H) at 5.52 ppm collapsed to a singlet on irradiation at the frequency of the proton ^{l}H as shown in Fig. III-9 (see also Fig. III-8). Thus the relationships between ^{k}H, ^{l}H and $C^{m}H_3$ was represented by partial structure (iv).



(iv)

B. Possible structures of the Piscicidal Constituent (**I**)

If the two tertiary methyl groups are placed on the partial structure (iiia) in geminal relationship, the partial structures (i~iv) described in section III–A remind us of phorbol (**III**) which has been isolated from *Croton tiglium,* a plant of the same family as *Sapium japonicum.*



phorbol (**III**)

Comparing the pmr spectrum of **I** in d_5-pyridine with that of phorbol,[39] all the protons (^{a}H~^{t}H) except two hydroxyl protons of the five in phorbol were detectable as shown in Table III–2. Therefore it was expected that **I** is a phorbol derivative. The appearence of the bands due to two ester carbonyls (1740 and 1725 cm^{-1}) in the ir spectrum of **I** together with the presence of three hydroxyl groups (cf. A–ii)) suggested that the piscicidal constituent (**I**) is one of phorbol-diesters. This was confirmed by lithium aluminum hydride reduction. The reduction of **I** with lithium aluminum hydride gave two alcohols **IV** and **V**, the former was derived from the alcohol moiety and the latter from the acid moiety. The alcohol (**IV**), mp 180~183 °C, was identified with phorbol,[31,39] which had been obtained on the reduction of phorbol–12,13,20–triacetate by L. Crombie, in respect of the pmr spectrum (Fig. III–10).

Table III–2. The pmr Spectral Data of **I** and Phorbol (**III**) in C_5D_5N.

H	**I** ppm	Phorbol (**III**) ppm	H	**I** ppm	Phorbol (**III**) ppm
a	3.64(1H, m)	3.66(1H, m)	k	5.86(1H, d, J_{kl}11)	4.98(1H, d, J_{kl}10–11)
b	7.69(1H, m)	7.81(1H, m)	l	2.78(1H, m)	2.75(1H, dq)
c	1.63(3H, dd)	1.68(3H, dd)	m	1.23(3H, d, J_{ml}5.5)	1.60(3H, d, J_{ml}6)
d, e	3.04(2H, s)	3.06(2H, s)	n	1.53(3H, s)	1.65(3H, s)
			o	1.26(3H, s)	1.57(3H, s)
f, g	4.30(2H, s)	4.26(2H, s)	p, s, t	4.9—5.4 (3H)	4.75, 5.37, 6.10 (each 1H, br)
h	5.5—6.9	6.10(1H, d, J_{hi}6)	q		7.73(1H, sharp)
i	3.92(1H, m)	3.90(1H, dd)	r		5.37(1H, sharp)
j	1.2—1.4	1.30(1H, d, J_{ji}5–6)			

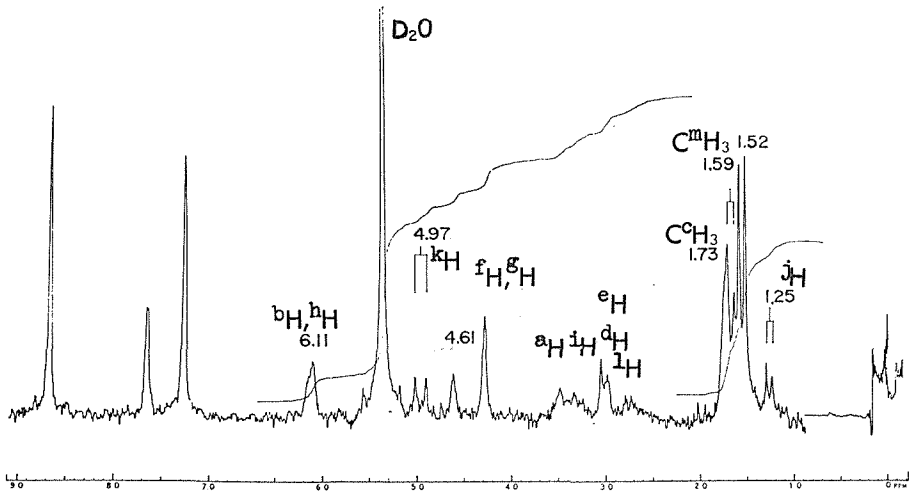
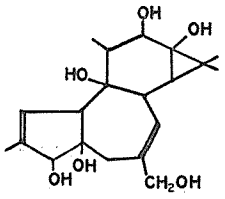


Fig. III–10. The pmr spectrum of IV(90 MHz, C_5D_5N).



IV

One of the acids which attached to phorbol through ester linkage is obviously acetic acid, because methyl protons (2.08 ppm) due to an acetoxyl group was observed in the pmr spectrum of **I**. The other acid, **VII**, was supposed to be a conjugated unsaturated acid according to the observation of ir absorption bands at 1725, 1260 and 1620 cm^{-1} (conjugated double bond) in **I**. The uv absorption at 307 nm suggested that it is a conjugated triene carboxylic acid.[40] The alcohol (**V**), obtained by the reduction of **I** with lithium aluminum hydride, showed the uv absorption at 260, 269, and 280 nm which are characteristic for triene. On hydrogenation over Adams catalyst, **V** gave a

saturated alcohol (**VI**) which was identified as *n*-decanol by its combined gas chromatography-mass spectrometry. Therefore, it was confirmed that **VII** is *n*-deca-2,4,6-trienoic acid.

On the basis of the foregoing a molecular formula, $C_{32}H_{42}O_8$, was able to be given for **I**, and the molecular weight of the formula satisfied the result of the mass spectrum (M^+ 554).

In the pmr spectrum of **I** in d_5-pyridine the doublet (5.86 ppm) due to ^kH appeared at a considerably lower field than that (4.98 ppm) of phorbol. Moreover, the doublet (^kH) in **I** was shifted upfield by 0.89 ppm to 4.97 ppm on the lithium aluminum hydride reduction (see Fig. III-10). These observations confirmed that one of *O*-acyl groups is attached to C–12 in **I**.

Hecker *et al.* had found that in phorbol analogs the tertiary hydroxyl group on the cyclopropane ring possessed the reducing activity against both Tollens and Fehling's reagents, and the activity was lost when the hydroxyl group was not free.[7] The compound (**I**) gave a negative Tollens test, indicating that another hydroxyl group to be acylated is the tertiary at C–13 of phorbol.

Thus the two possible structures (**Ia** and **Ib**) are proposed for **I** as illustrated in Fig. III-11.

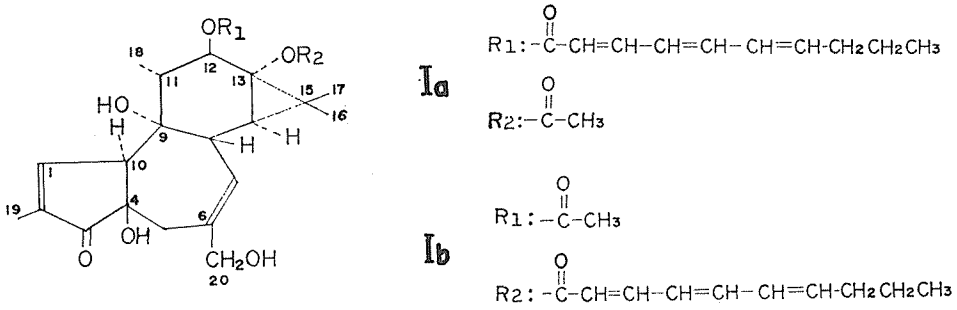
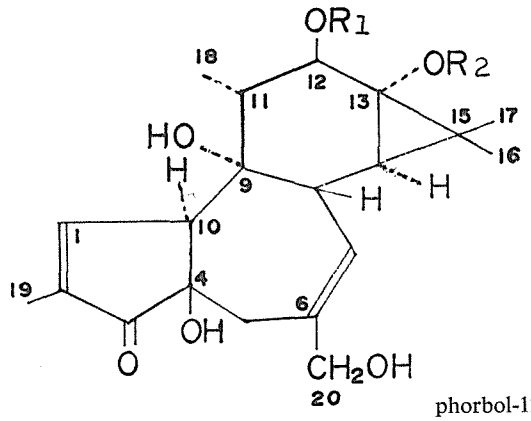


Fig. III–11. The possible structures of **I**.

C. Structure of **I**

Up to date 11 kinds of phorbol-12,13-diesters, about which additional discussions



phorbol-12,13-diesters

will be found in section III–4, have been isolated from *Croton tiglium*.[4~6] They provided significant informations for structural elucidation of **I**.

In the mass spectra of the phorbol-12,13-diesters, characteristic fragment peaks corresponding to M-$R_1COO\cdot$, and M-R_2COOH has been observed.[41] In the mass spectrum of **I** (Fig. III-3), the significant peaks were observed at *m/e* 494 and 389. The peak at *m/e* 494 (M–60) was likely to be a fragment in which acetic acid was lost from **I**. The peak at *m/e* 389 (M–165) was attributed to a fragment formed by the elimination of *n*-deca-2,4,6-trienoyloxy radical ($C_9H_{13}COO\cdot$). The schema of significant fragmentations of **I** in the mass spectrometry is given in Fig. III-12. The observation of these fragments suggested that the acetoxyl group is attached to C–13.

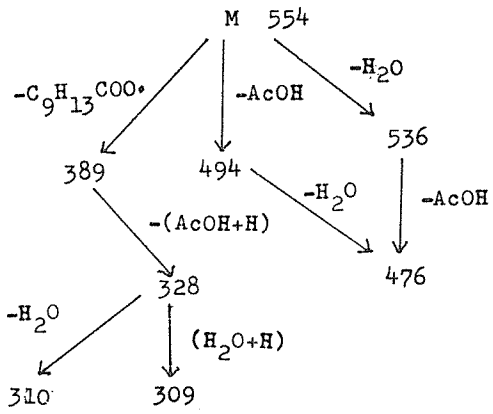


Fig. III–12. The schema of significant fragmentations of **I** in the mass spectrometry.

Further suggestion for this structural feature was given by selective deacylation at C–13. Hecker *et al.* has reported that phorbol-12-monoesters were formed from phorbol-12,13,20-triesters by transesterification with a trace of base.[41] A small quantity of **I** was submitted to the reaction and the reaction was followed by thin layer chromatography of silica gel GF_{254}. As the reaction proceeded, a new spot which had lower *Rf* value than **I** was detected on the chromato plate together with the spot due to **I**. Under the normal acetylation condition the reaction products formed a single derivative which was confirmed as monoacetate (**II**) by co-thin layer chromatography. These results are illustrated in Fig. III-13 and indicated that **I** was deacetylated at C–13 with a trace of base to yield a phorbol-12-monoester (**VIII**), and on the treatment of **VIII** with acetic anhydride-pyridine, the hydroxyl group at C–13 of **VIII** together with the hydroxyl group at C–20 was acetylated to give a monoacetate (**II**) of **I**. The reaction pathways to be expected are shown Fig. III-14.

The observations mentioned above supported strongly that the structure of the piscicidal constituent (**I**) is represented by the formula **Ia** rather than **Ib** apart from the stereochemistry.

If the structure of **I** is **Ia**, hydrogenation of the conjugated triene moiety of **I** must lead to an authentic 12-*O*-*n*-decanoyl-phorbol-(13)-acetate, which has been isolated from *Croton tiglium*.[41]

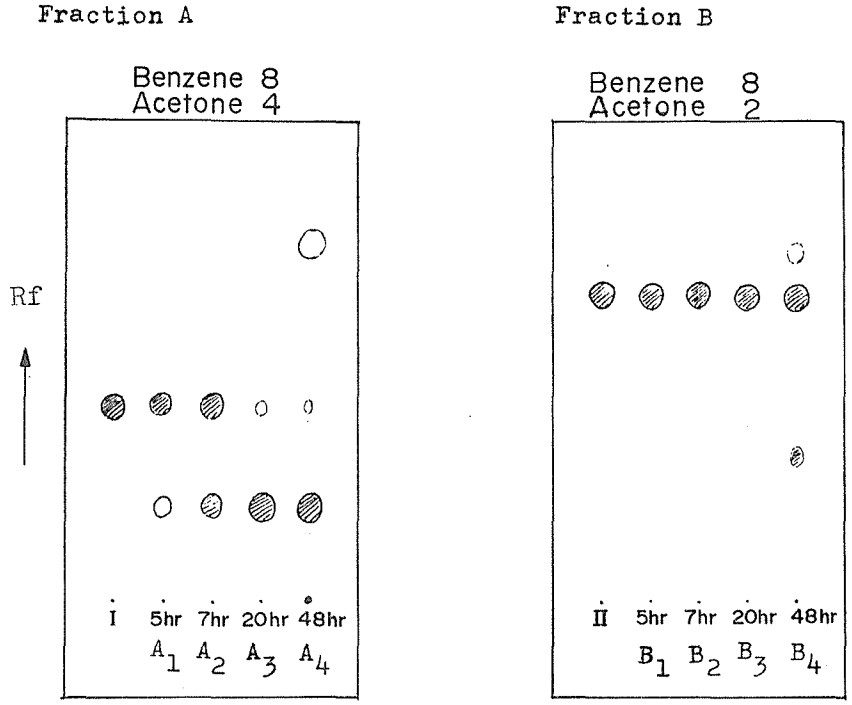


Fig. III–13. Thin layer chromatograms of products on transesterification and acetylation.
Fraction A_1~A_4: fractions obtained by transesterification of I.
Fraction B_1~B_4: fractions obtained by acetylation of Fraction A_1~A_4.

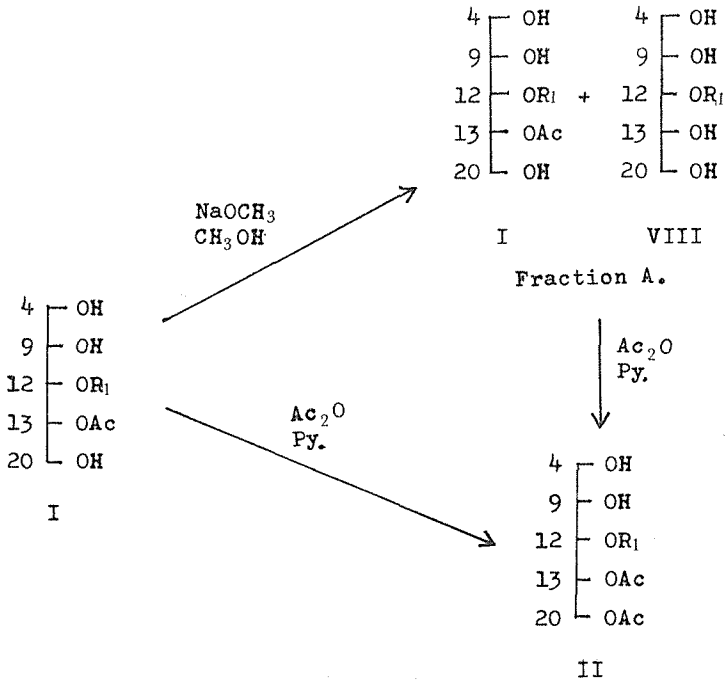
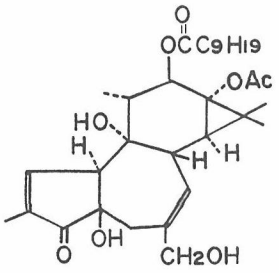


Fig. III–14. Attempted transesterification of I.



IX

On the restricted catalytic hydrogenation, **I** afforded a compound (**IX**). The mass spectrum of **IX** showed a molecular ion peak at m/e 560, indicating that **I** absorbed three molar equivalents of hydrogen successfully. The hexahydro derivative (**IX**), showed the uv absorption maximum in ethanol at 232 nm (4.83×10^3), and the ir (KBr pellet) (Fig. III–15) absoption bands at 3100~3600 (broad), 1740~1690 (broad),

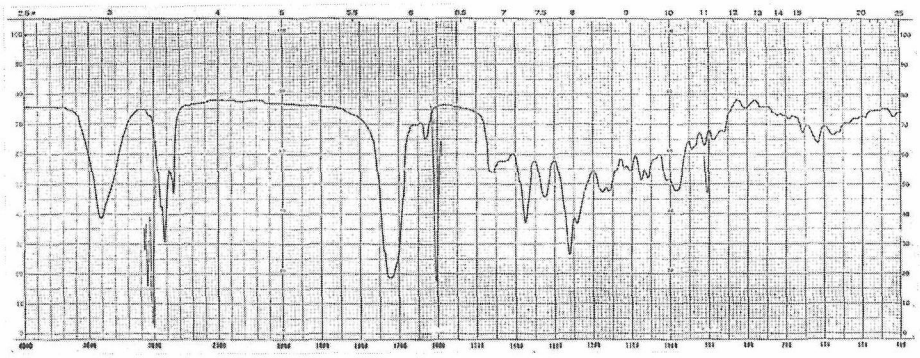


Fig. III–15. The ir spectrum of **IX** (KBr pellet).

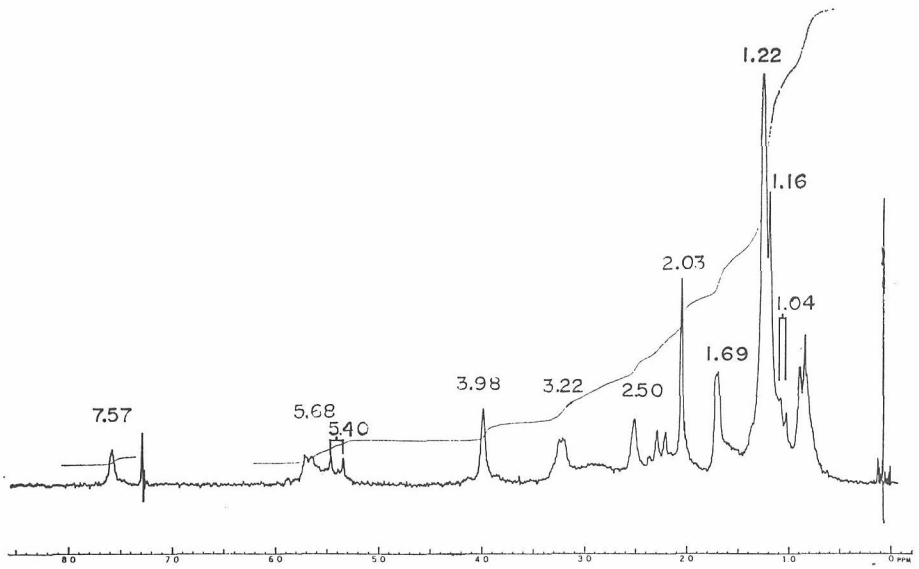


Fig. III–16. The pmr spectrum of hexahydro derivative (**IX**) (90 MHz, $CDCl_3$)

1630, and 1260 cm^{-1}. In its pmr spectrum (Fig. III-16) most of olefinic proton signals disappeared, whereas the broad signal corresponding to methylenes newly appeared near at 1.2 ppm. These spectroscopic data explained that the conjugated system except for the enone system of **I** was hydrogenated. And the data including the optical rotation, $[\alpha]_D^{17.0}$ +57.3° (*c* 0.75, dioxane), were identical with those of 12-*O*-*n*-decanoyl-phorbol-(13)-acetate.

The structure of the piscicidal constituent (**I**) was thus confirmed except for the configuration of the conjugated triene moiety and is represented by the formula **Ia**, 12-*O*-*n*-deca-2,4,6-trienoyl-phorbol-(13)-acetate.

III-3. Piscicidal Activities of I and Its Derivatives

The piscicidal activities of **I** and its derivatives were evaluated with killie-fish (*Oryzias laptipes*),[25] and the experimental data of **I** are shown in Table III-3. The 24- and 48-hr median tolerance limits of **I** and its derivatives, which were estimated by straight line graphycal interporation, are listed in Table III–4, together with those of rotenone,[23] callicarpone,[23] huratoxin[25] and sodium pentachlorophenoxide.[25]

Table III–3. Piscicidal Activity of **I** to Killie-fish

Conc. of **I** (ppm)	Number of test fish added originally	Number of test fish surviving after 24 hr	48 hr
0.00656	5	0	0
0.00328	5	0	0
0.00262	5	4	1
0.00197	5	5	4
0.00131	5	5	5
0.000656	5	5	5

Table III–4. Piscicidal Activity TLm.

	24 hr		48 hr	
	ppm	μM	ppm	μM
Active Compound (I)	0.0030	0.0054	0.0023	0.0042
Monoacetate (II)	0.0016	0.0027	0.0014	0.0023
Hexahydro derivative (IX)	0.0041	0.0073	0.0041	0.0073
Phorbol (III)	>1		>1	
Phorbolol (IV)	>1		>1	
Rotenone*	0.013	0.033	0.012	0.030
Callicarpone*	0.042	0.13	0.024	0.072
Huratoxin	0.0014	0.0024	0.0011	0.0019
PCP-Na*	0.25	0.87	0.24	0.83

* The test condition is different from that of active compound *etc.*, the test solution in the case of rotenone *etc.* is composed of 10 liter of water and an acetone solution (1 ml) of a test compound of a known concentration (in the case of PCP-Na, methanol solution was used).

The piscicidal constituent (**I**) was about 4 times as toxic as rotenone, and the exceedingly strong toxicity of **I** is comparable to that of huratoxin (**X**), which have been isolated from *Hura crepitans*, a plant of the same family (Euphorbiaceae) as *S. japonicum*, and whose diterpene part is similar to that of **I**.

The monoacetate (**II**) and the hexahydro derivative (**IX**) showed as much piscicidal activity as **I**. Phorbol (**III**) and phorbolol (**IV**) exhibited, however, no more activity against killie-fish. As judged by these results, the presence of *O*-acyl groups at both C–12 and C–13 of phorbol is required to exhibit the piscicidal activity. And these groups probably play a significant role in the penetrating of the molecule into cells.

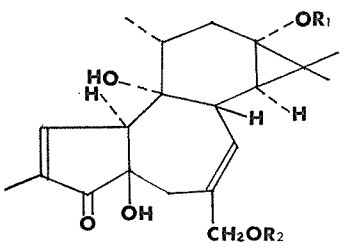
Interestingly, the requirement for the piscicidal activity of **I** is comparable to that for tumor promoting and inflammatory activities of phorbol-12,13-diesters, reported by Hecker *et al.*[4,5] Therefore it is expected that **I**, as well as the other phorbol-12,13–diesters, has also tumor promoting and inflammatory activities.

III-4. A Brief Review of Phorbol-esters and Related Compounds

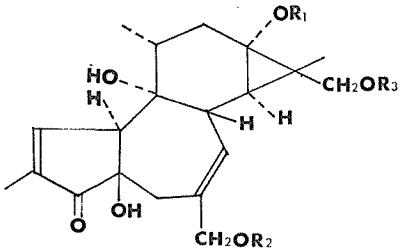
As stated breefly in the preceding chapters, Hecker *et al.* have investigated the constituents of tumor promoting and inflammatory substances of *Croton tiglium,* and isolated 11 kinds of phorbol-12,13-diesters as the active principles (Table III–5).[5,6] They have also screened other plants in the *Euphorbia* species for the tumor promoting and inflammatory activities, and have isolated 6 kinds of 12-desoxy-phorbol esters (**XI**~**XVI**),[42] 2 kinds of 16-hydroxy-12-desoxy-phorbol esters (**XVII, XVIII**),[43] and ingenol ester (**XIX**)[44] from *Euphorbia triangularis, Euphorbia cooperi* and *Euphorbia ingens* respectively as active principles.

Table III–5. Phorbol-12,13-diesters Isolated from *Croton tiglium.*

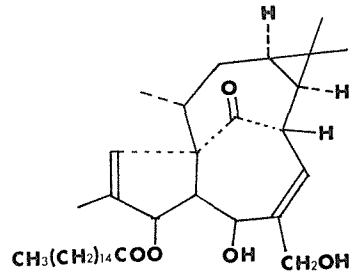
ester	acid residue (C–12)	acid residue (C–13)
A_1	myristic	acetic
A_2	capric	acetic
A_3	lauric	acetic
A_4	palmitic	acetic
B_1	(+)-*S*-2-methylbutyric	lauric
B_2	(+)-*S*-2-methylbutyric	capric
B_3	tiglic	capric
B_4	acetic	lauric
B_5	(+)-*S*-2-methylbutyric	caprylic
B_6	tiglic	caprylic
B_7	acetic	capric



12-desoxy-phorbol esters
(**XI**~**XVI**)

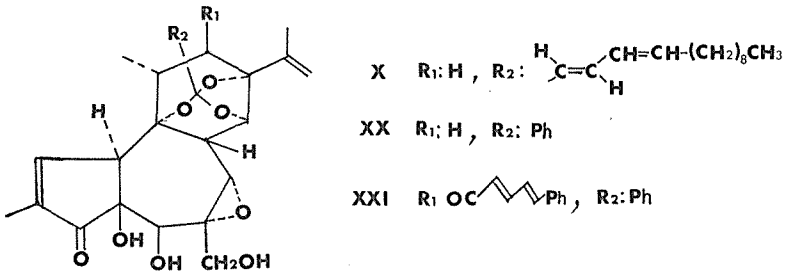


16-hydroxy-12-desoxy-
phorbol esters (**XVII, XVIII**)



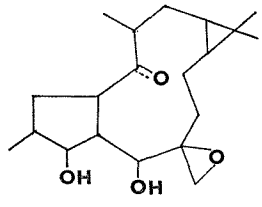
ingenol ester (XIX)

Recently huratoxin (X) has been isolated from *Hura crepitans* (Euphorbiaceae).[25] The carbon skeleton of the diterpene part of huratoxin is very similar to that of phorbol. It is very interesting that the phorbol diesters and related compounds which exhibit toxicity are distributed widely among plants in the family, Euphorbiaceae. And other phorbol diesters and related compounds with toxicity are expected to occur in plants belonging to Euphorbiaceae.

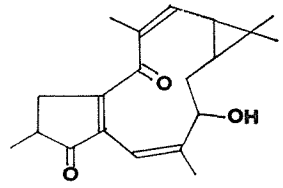


On the other hand daphnetoxin (XX)[45] and mezerein (XXI)[46,47] have recently been isolated from *Daphne mezereum,* a plant belonging to Thymelaeaceae as toxic principles. The occurrence of the same diterpene alcohol in Euphorbiaceae and Thymelaeaceae may give additional support for a close relationship between the two families.

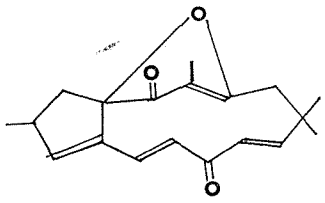
The carbon skeleton of the diterpene part of the toxic compounds stated above are composed of 5,7,6-membered ring system except for XIX. This skeleton have been unable to be explained in terms of the usual biogenetic route from geranyl-geranyl pyrophosphate established for common bi-, tri- or tetracyclic diterpenes. It is noteworthy that macrocyclic diterpenes, 6,20-epoxy-lathyrol (XXII),[48,49] 7-hydroxy-lathyrol (XXIII),[50] bertiyadionol (XXIV)[51] and jatrophone (XXV)[52] have been isolated from



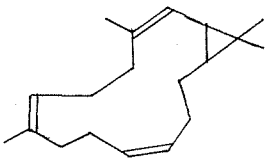
XXII



XXIV



XXV



XXVI

Euphorbia lathyris, a *Bertya* sp. *nov.* and *Jatropha gossypiifolia* (Euphorbiaceae) apart from biological activity. The diterpenes composed of 5,11- or 5,12-membered carbocyclic system are probably precursors of the diterpenes composed of 5,7,6-membered ring system.

On the other hand Robinson and West have recently succeeded in the biosynthesis of casbene (XXVI), a macrocyclic diterpene composed 14-membered ring system, as well as of the known cyclic diterpenes such as (+)-beyerene, (−)-kaurene, *etc.*, using the extracts from the seedlings of castor bean, and proposed a hypothetical biogenetic pathway of casbene from geranyl-geranyl pyrophosphate.[53]

On the basis of the foregoing, the carbon skeleton composed of 5,7,6-membered ring system is assumed to be formed by recyclization of macrocyclic diterpenes such as casbene.

It is expected that further investigations of chemical constituents of plants in Euphorbiaceae may reveal the biogenetic route of diterpenes composed 5,7,6-membered ring system.

III-5. Experimental

Infrared spectra were recorded on a Hitachi Model EPI-G3 spectrophotometer and calibrated with 3027.1, 1601.5 and 906.7 cm^{-1} bands of polystyrene. Proton magnetic resonance (pmr) spectra were recorded on a Hitachi Model R-22 spectrometer (90 MHz). Optical rotations were measured with a Yanagimoto photomagnetic direct reading polarimeter Model OR-20. Ultraviolet spectra were determined with a Hitachi EPS-3T spectrophotometer, and mass spectra with a Hitachi RMU-6D mass spectrometer and Hitachi RMU-6L mass spectrometer at 70 eV. Gas chromatography was performed on a Hitachi Model 063 instrument with a F.I.D. detector. In combined gas chromatography-mass spectrometry, a Hitachi K-53 gas chromatograph with a T.C.D. detector and a Hitachi RMS-4 mass spectrometer were used. Melting points were determined on a hot stage, and are uncorrected. The following chromatographic materials were used: silicic acid (Mallinckrodt, 100 mesh, U.S.A.), Florisil (Floridin Company, 100~200 mesh), silica gel G and GF_{254} (Merck, for thin layer chromatography) and silica gel PF_{254} (Merck, for preparative thin layer chromatography), granular charcoal and charcoal (Wako Pure Chem., Tokyo). Celite 545 washed successively with distilled water and acetone, and then dried at 100°C for 5 hr before use.

Isolation of the piscicidal constituent (I)

The young twigs (133 kg) of *Sapium japonicum* were extracted with methanol

(24×20 liters), and the methanol extracts were concentrated *in vacuo*, and the aqueous concentrate (24 liter) was extracted with benzene (2×24 liters) followed with ethyl acetate. The piscicidal activity was found in the benzene extracts. The extract was dried over anhydrous sodium sulfate and evaporated to yield dark green residue (614 g). A part (24.5 g) of this residue was adsorbed on Celite 545 (45 g) and placed on the top of a column of granular charcoal (230 g). The column was eluted in 1.5 liter fractions of water containing increasing amounts of acetone (20, 40, 60, 80, 90 and 100%) and of 100% benzene. The column chromatography of the same scale was performed 25 times, and the activity was found in the fraction eluted with 100% acetone. The combined eluate (68.3 g) was chromatographed on Florisil 20 times as much as the sample by 5% stepwise elution from benzene to ethyl acetate. The fraction eluted with 30% ethyl acetate in benzene was further purified by silicic acid-Celite 545 column chromatography with 2.5% stepwise elution from *n*-hexane to acetone. The active fractions eluted with 17.5 and 20% acetone in *n*-hexane were combined and separated by silicic acid-Celite 545 column chromatography with 5% stepwise elution from benzene to ethyl acetate. The active fractions eluted with 25, 30 and 35% ethyl acetate in benzene were finally chromatographed on charcoal, and the column was eluted with methanol containing an increasing ratio (10% step) of acetone. The fractions (30, 40 and 50% acetone in methanol) gave the piscicidal constituent (**I**) in a total yield of 349 mg as a colorless glassy resin.

$[\alpha]_D^{28.5}$ −19.6° (*c* 0.88, CH_3OH);

MS:*m/e* 554 (M^+);

UV λ_{max}^{EtOH} nm(ε):232 (9.24×10^3, sh.), 307 (3.59×10^4);

IR $\nu_{max}^{CHCl_3}$ cm^{-1}:3500~3200 (broad), 1740 (sh.), 1725, 1710, 1620 and 1260;

PMR: ($CDCl_3$), 0.87 (3H, d., *J*=6), 0.89 (3H, t., *J*=6), 1.07 (1H, d., *J*=5), 1.20 (3H, s.), 1.23 (3H, s.), 1.2~1.5 (2H, m.), 1.72 (3H, broad s.), 2.08 (3H, s.), 2.0~2.4 (5H, m.), 2.52 (2H, s.), 3.1~3.4 (1H+1H, m.), 3.99 (2H, s.), 5.46 (1H, d., *J*=11), 5.5~7.5 (8H~9H), 7.60 (1H, broad s.);

(CD_3COCD_3), 0.86 (3H, d., *J*=6), 0.88 (3H, t., *J*=6), 1.14 (1H, d., *J*=5), 1.20 (3H, s.), 1.27 (3H, s.) 1.2~1.6 (2H, m.), 1.67 (3H, m.), 2.50 (2H, s.), 3.1~3.4 (1H+1H, m.), 3.7~3.9 (OH, broad), 3.94 (2H, s.), 4.72 (OH, s.), 5.26 (OH, s.), 5.54 (1H, d., *J*=11), 5.6~7.4 (7H), 7.53 (1H, broad s.);

($CDCl_3+C_6D_6$, 2:1), 0.87 (3H, t., *J*=6), 0.90 (3H, d., *J*=6), 1.05 (1H, d., *J*=5), 1.17 (3H, s.), 1.24 (3H, s.), 1.2~1.5 (2H, m.), 1.67 (3H, m.), 2.00 (3H, s.), 1.9~2.3 (4H, m.), 2.47 (2H, s.), 3.1~3.4 (1H+1H, m.), 3.91 (2H, s.), 5.52 (1H, d., *J*=11), 5.5~7.4 (7H), 7.58 (1H, broad s.);

(C_5D_5N), 0.76 (3H, t., *J*=6), 1.23 (3H, d., *J*=5.5), 1.26 (3H, s.) 1.1~1.5 (2H+1H, m.), 1.53 (3H, s.), 1.63 (3H, m.), 1.7~2.1 (2H, m.), 2.09 (3H, s.), 2.78 (1H, m.), 3.04 (2H, s.), 3.64 (1H, m.), 3.92 (1H, m.), 4.30 (2H, s.), 4.9~5.4 (OH), 5.86 (1H, d., *J*=11), 5.5~7.2 (7H), 7.69 (1H, m.).

Acetylation of ***I***

The piscicidal constituent (**I**) (28 mg) was treated with acetic anhydride (3 ml) in pyridine (1.5 ml) at room temperature overnight. The reaction mixture was poured into ice-water and extracted with ethyl acetate. The ethyl acetate layer was washed several times with water, dried over anhydrous sodium sulfate, and the solvent was evaporated. The colorless glassy residue (28 mg) thus obtained was purified by silicic acid-Celite 545 column chromatography eluted with benzene containing an increasing ratio (2.5% step) of ethyl acetate to yield a monoacetate (**II**) (20 mg) as a colorless glassy resin.

$[\alpha]_D^{20}$ $-16.5°$ (*c* 0.95, $CHCl_3$);

UV λ_{max}^{EtOH} nm (ε):232 (1.07×10^4, sh.), 307 (3.22×10^4);

IR $\nu_{max}^{CHCl_3}$ cm^{-1}:3600~3100, 1740 (sh.), 1725 (sh.), 1710, 1615, 1170, 1000;

MS:*m/e* 596 (M^+);

PMR: ($CDCl_3$), 0.88 (3H, d., *J*=6), 0.89 (3H, t., *J*=7), 1.05 (1H, d., *J*=5), 1.20 (3H, s.), 1.23 (3H, s.), 1.2~1.6 (2H, m.), 1.74 (3H, m.), 2.03 (3H, s.), 2.10 (3H, s.), 2.1~2.4 (2H+1H+1H), 2.49 (2H, broad s.), 3.27 (2H, m.), 4.48 (2H, s.), 5.50 (1H, d., *J*=11), 5.6 (1H, broad s.) 5.6~7.5 (olefinic protons, 7H), 7.64 (1H, broad s.).

Lithium aluminum hydride reduction of ***I***

A suspension of lithium aluminum hydride (40 mg) in dry ether (3 ml) was added dropwise into the solution of **I** (55 mg) in dry ether (7 ml) with stirring under 0°C. After the reaction mixture was refluxed for 2 hr, it was cooled to 0°C. A small quantity of water was added into the reaction mixture, and extracted with ether.

The aqueous layer was adjusted to pH 7, and the solvent was evaporated *in vacuo* to give a residue (112 mg), which was extracted with hot ethanol several times. The filtered extracts were evaporated to dryness to give a solid (46 mg). The solid was purified by preparative thin layer chromatography on silica gel PF_{254} developed with a solvent ($CHCl_3$:CH_3OH, 8:2) to yield a crystalline matter. Recrystallization from ethyl acetate gave an alcohol (**IV**) (27 mg) as colorless plates, mp 180—183°C.

UV λ_{max}^{EtOH} nm (ε):210 (end absorption, 1.98×10^4);

IR ν_{max}^{Nujol} cm^{-1}:3600~3100, 1660, 1460, 1380;

PMR:(C_5D_5N), 1.25 (1H, d., *J*=5), 1.52 (3H, s.), 1.59 (3H, s.), 1.68 (3H, d., *J*=6), 1.73 (3H, m.), 3.0 (2H, broad s.), 2.75 (1H, m.), 3.30~3.60 (1H+1H, m.), 4.29 (2H, s.), 4.61 (1H, broad s.), 4.97 (1H, d., *J*=11), 4.8 (6×OH), 6.11 (2H, broad s.).

The ether extract was washed with water several times, dried over anhydrous sodium sulfate and the solvent was evaporated to yield a colorless residue. Puryfying this residue by silicic acid-Celite 545 (1:1) column chromatography eluted with benzene containing an increasing ratio of ethyl acetate (5% step), an alcohol (**V**) (3.6 mg) was obtained as a colorless oil.

UV $\lambda_{max}^{n\text{-hexane}}$ nm(ε):260 (1.95×10^4), 269 (2.52×10^4), 280 (1.95×10^4).

Catalytic hydrogenation of ***V***

The alcohol **V** (3.0 mg) in ethanol (5 ml) was hydrogenated over Adams catalyst until no more hydrogen was absorbed. The catalyst was filtered off and the solvent

was removed *in vacuo*, to give **VI** as a colorless oil. It was identified as *n*-decanol by measurement of its retention time on the gas chromatography (G.L.C) and by its combined gas chromatography-mass spectrometry (GC-MS). **VI** was chromatographed on the column (stainless steel tube 1 m×3 mm i.d.) packed with 5% PEG on Celite 545 at 100°C with a helium flow rate of 30 ml/min and gave a retention time 18.5 min, which was identical with that of an authentic *n*-decanol. In GC-MS a stainless steel column (1 m×3 mm i.d.) packed with 5% PEG 20M on 80~100 mesh Chromosorb W.AW.HMDS. was used, and **VI** was chromatographed on the column at 110°C with a helium flow rate of 30 ml/min and peak at retention time 8.3 min was scanned for *m/e* 10~200 at a source temp. of 150°C.

MS:*m/e* (relative intensity) 140 (M–18) (6.8), 85 (33.0), 84 (46.6), 71 (79.6), 70 (68.0), 69 (29.1), 57 (46.6), 56 (91.3), 55 (99.0), 43 (100), 41 (89.3).

Attempted base catalized transesterification of ***I***

Three ml of 10^{-3}% methanolic sodium methoxide solution was added into a solution of **I** (2 mg) in absolute methanol (10 ml) and allowed to stand under anhydrous condition. One ml of reaction mixture was pipetted into water (2 ml) every certain hours (5 hr., 7 hr., 20 hr. and 48 hr.). A reaction mixture diluted was concentrated *in vacuo* and the aqueous concentrate was extracted with ethyl acetate. The ethyl acetate extract was washed several times with water, dried over anhydrous sodium sulfate to give a residue, tentatively named as fraction A_1. The mixtures obtained every certain hours were worked up by the same way as the above to give fractions A_2—A_4. A portion of each fraction was examined by thin layer chromatography, and the fraction remained was submitted to usual acetylation. On the acetylation reaction product tentatively named as fraction B_1, was obtained from A_1, B_2, B_3 and B_4 from A_2, A_3 and A_4 respectively. And the fractions B_1, B_2, B_3 and B_4 were also examined by thin layer chromatography.

Preparation of ***III***

To a solution of **I** (30 mg) in absolute methanol (10 ml) was added 5 ml of 5% methanolic sodium methoxide solution and allowed to stand overnight under anhydrous condition. The reaction mixture was poured into ice-water (2 ml). After the pH was adjusted to 7, the solvent was evaporated *in vacuo* to yield colorless solid (102 mg). It was chromatographed on a column of silicic acid Celite-545 and the column was eluted with ethyl acetate containing increasing amounts of methanol (0, 1, 2, 3, ...10, 15, 100%). A crystalline matter was obtained in the fraction eluted 4, 5, 6% methanol in ethyl acetate. Recrystallization from ethyl acetate to yield phorbol (**III**) (6.7 mg) as colorless prisms, mp 240—244° (decomp.).

IR ν_{max}^{KBr} cm^{-1}: 3700~3000, 1700, 1640;

MS:*m/e* 346 (M–18);

PMR: (C_5D_5N) 1.31 (1H, d., J=6), 1.58 (3H, s.), 1.61 (3H, d., J=6), 1.67 (3H, s.), 1.69 (3H, broad s.) 3.09 (2H, s.), 3.69 (1H, m.), 3.96 (1H, m.) 4.30 (2H, s.), 5.05 (1H, d., J=11), 6.17 (1H, m.) 7.89 (1H, m.).

Catalytic hydrogenation of ***I***

The active constituent (**I**) (33 mg) in ethanol (10 ml) was hydrogenated over Adams catalyst (8 mg). The reaction was continued at atmospheric pressure and at room

temp. until 3 molar equivalents of hydrogen was consumed. The catalyst was filtered off and the filtrate was evaporated to yield a resinous matter (32 mg). It was chromatographed on charcoal eluted with methanol containing an increasing ratio of acetone. A hexahydro derivative (IX) (26 mg) was obtained in the fraction eluted 30% acetone in methanol as a colorless glassy resin.

$[\alpha]_D^{17.0}$ +57.3° (*c* 0.75, dioxane);

UV λ_{max}^{EtOH} nm (ε):232 (4.83×10^3);

IR ν_{max}^{KBr} cm^{-1}:3600～3100 (broad), 1740～1690 (broad), 1630, 1260;

MS:*m/e* 560 (M^+);

PMR: ($CDCl_3$), 0.82 (3H, t., *J*=6), 0.84 (3H, d., *J*=6), 1.04 (1H, d., *J*=6), 1.16 (3H, s.), 1.22 (17H), 1.69 (3H, m.), 2.03 (3H, s.), 2.24 (2H, m.), 2.50 (2H, broad s.), 2.6～3.1 (2×OH), 3.22 (1H+1H, m.), 3.98 (2H, s.), 5.40 (1H, d., *J*=11), 5.4～5.7 (1×OH), 5.68 (1H, m.), 7.57 (1H, broad s.).

Piscicidal test

Oryzias laptipes (killie-fish) averaging 350 mg in weight and 3～3.5 cm in length were used as the test fish, which were not fed for two days before they were used in a test. As the test containers 200 ml beakers served, in which 150 ml of water was kept. The water must be aerated by means of an air-pump for a few hours. An acetone solution (0.5 ml) of a test compound of a known concentration was added with vigorous stirring. Five test fish were introduced to the test solution. The solution prepared by adding only 0.5 ml of acetone served as control. Fish having died during the test were immediately removed from the solution and those which have been surviving in each test container were observed and recorded exactly 24- and 48-hr after their introduction. The experimental data are listed in Tables III-3 and III-4. The median tolerance limits were estimated by a straight-line graphical interpolation using survival percentages at two successive concentration of the test series which were lethal to more than half and to less than half of the test fish. The experimental data for toxicity of callicarpone, rotenone, huratoxin and sodium pentachlorophenoxide to the killie-fish are listed for comparison with that of I in Table III-4.

ACKNOWLEDGMENTS

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IV. STUDIES ON AN ANTIFUNGAL CONSTITUENT OF *SAPIUM JAPONICUM*

IV-1. A Brief Review of Naturally Occurring Antifungal Substances in Plants

There have been many examples of utilizing natural products to protect microbial

harms. Antibiotics are one of the most prominent examples. Some plants have also been used in remedies, antiseptics and so on.

The resistance of plants to disease may be due to the presence of preformed natural fungicides, or to the synthesis of these compounds (phytoalexins) in the plant tissue in response to fungal or viral infection.[54] It has been suggested that further identification of the structures and properties of natural protective agents should lead to the recognition of new, effective, and perhaps, safer agricultural fungicides.[55] With this objective in mind, screening of some plant extracts for antifungal properties have been carried out for these past 20 years.

Goto *et al.* screened 400 species of plants against several micro organisms and reported that the plants belonging to the family Araliaceae, Berberidaceae, Betulaceae, Caprifoliaceae, Ericaceae, Hippocastanaceae, Leguminosae, Pittosporaceae, Ranunculaceae, Rosaceae, Saxifragaceae, Theaceae, Eupteleaceae, Dioscoreaceae, Liliaceae showed moderately remarkable activity.[56]

Y. L. Nene *et al.* also screened 88 species of plants for antifungal properties against *Helminthosporium turcicum* Pass., and reported 14 species were available for antifungal properties.[57]

The active constituents with antifungal activities are classified as follows: carboxylic acids, amino acids, phenolic compounds, phenolic acids, lactones and coumarins, tannins, acetylenic compounds, quinones, tropolones, benzoxazolinones and so on.

IV-2. Extraction and Isolation of an Antifungal Constituent of *Sapium japonicum*

The extraction and isolation procedure was controlled by the inhibitiory effect against conidia germination of *Cochliobolus miyabeanus*[58] and is illustrated in Fig. IV-1. The ethyl acetate-soluble fraction of methanol extracts obtained from the fresh leaves (4.0 kg) was chromatographed on silicic acid-Celite 545 column and eluted

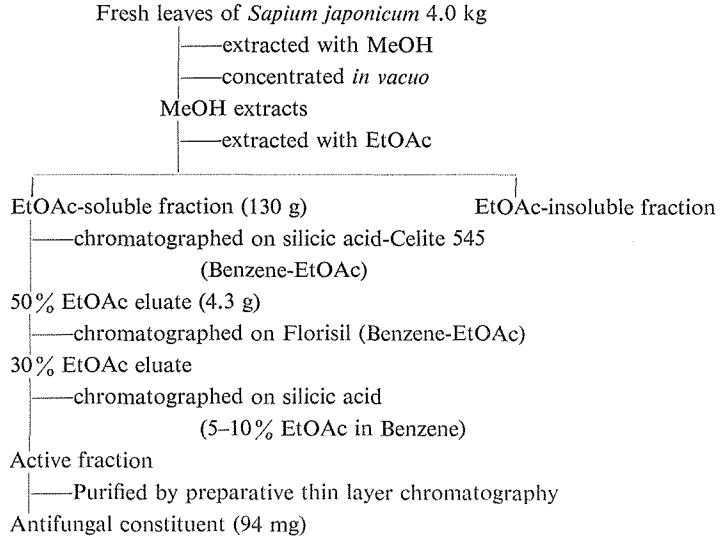


Fig. IV-1. Extraction and Isolation Procedure.

stepwise with benzene containing an increasing ratio of ethyl acetate. The activity was found in the fraction eluted with 50% ethyl acetate. This fraction was purified by adsorption column chromatographies of Florisil and silicic acid-Celite 545, followed by the preparative thin layer chromatography. An antifungal constituent (**1**) was obtained as a colorless oil in a total yield of 2.4×10^{-3}% from the fresh leaves. The homogeneity of the compound was established by thin layer chromatography on silica gel G (benzene-ethyl acetate 4:1, *Rf* 0.4, benzene-acetone 7.5:2.5, *Rf* 0.3). The antifungal constituent (**1**) was visualized by characteristic reddish brown coloration when sprayed with 4% ammonium metavanadate in 50% sulfuric acid. It readily decomposes itself under exposing in the air.

The antifungal constituent (**1**), $[\alpha]_D^{12}$ −51.3° (*c* 0.94, $CHCl_3$) showed uv absorption maxima (EtOH) at 219 (ε 1200) and 244 nm sh. (ε 710). The ir spectrum (Fig. IV-2) shows bands at 3400, 1730 and 1160 cm^{-1}. In addition a characteristic intense band is observed at 1965 cm^{-1}. The pmr spectrum of **1** (60 MHz, $CDCl_3$) (Fig. IV-3) shows the signals at 1.6~2.2 ppm (4H, multiplets), 2.38 ppm (2H, triplet, *J*=6 Hz), 3.68

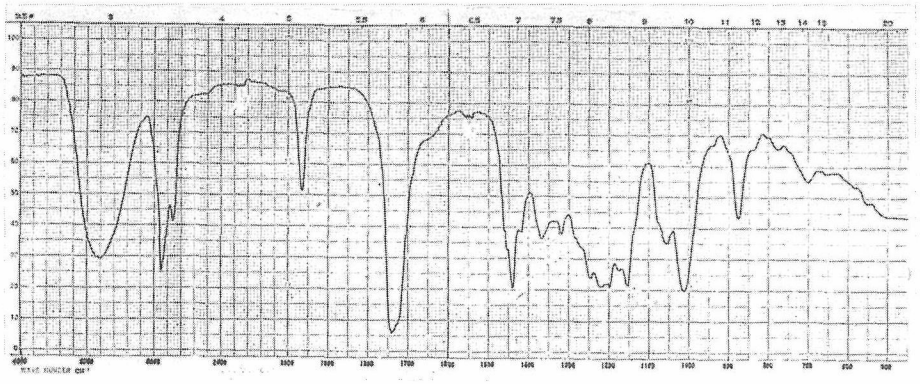


Fig. IV–2. The ir spectrum of **1** (Liquid).

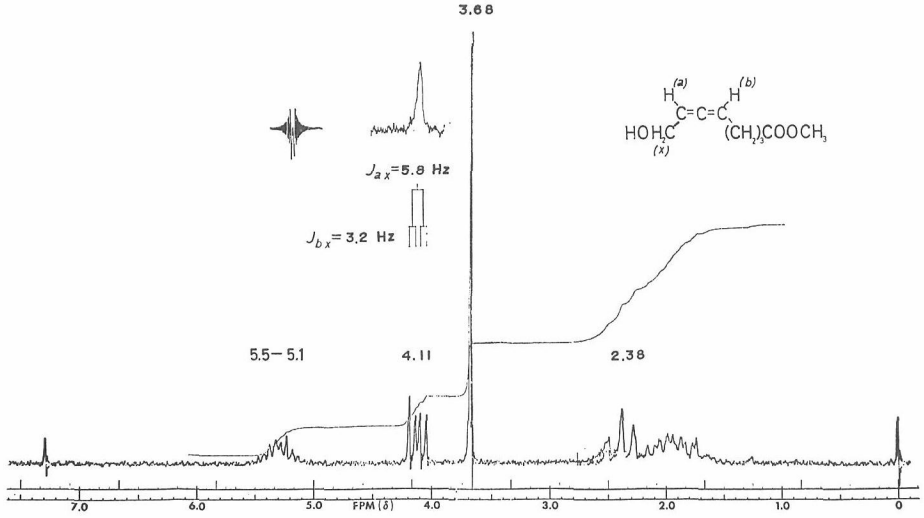


Fig. IV–3. The pmr spectrum of **1** (60 MHz, $CDCl_3$).

ppm (3H, singlet), 4.11 ppm (2H, double doublet, J=5.8 Hz, 3.2 Hz) and 5.1~5.5 ppm (2H, multiplets).

IV-3. Chemical Structure of an Antifungal Constituent (1)

A. Functional groups

i) The presence of a methoxyl carbonyl group

The ir absorption band at 1730 and 1160 cm^{-1} is indicative of an ester group. A sharp three-proton singlet at 3.68 ppm in the pmr spectrum suggested the presence of a methoxyl carbonyl group.

ii) The presence of a non-terminal disubstituted allene

The characteristic ir absorption band at 1965 cm^{-1} [59] and the pmr signals at a region of 5.1~5.5 ppm suggested the presence of an allene. Hydrogenation of **1** over Adams catalyst gave a tetrahydro derivative (**2**), whose ir spectrum (Fig. IV-4) showed

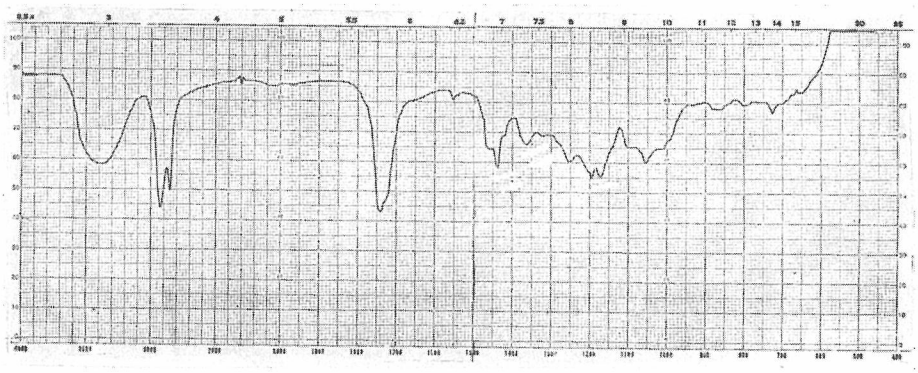


Fig. IV-4. The ir spectrum of **2** (Liquid).

no band at a region of 1965 cm^{-1}. The signals at the region of 5.1~5.5 ppm in the pmr spectrum of **1** were absent in **2**. These results indicated the presence of an allene. Furthermore, attention had to be given to the fact that **2** is optically inactive. This means that the optical rotation of **1** is based only on the asymmetry due to the allenic double bond which is not located at terminal. Thus the presence of a non-terminal disubstituted allene was confirmed.

iii) The presence of a primary hydroxyl group

On acetylation with acetic anhydride and pyridine, **1** gave a monoacetate (**3**). In its pmr spectrum a two-proton singlet at 4.11 ppm was shifted downfield by 0.45 ppm to 4.56 ppm. This indicated that the original hydroxyl group was primary.

B. Determination of the molecular formula

Although the mass spectrum of the monoacetate (**3**) did not show clearly the molecular ion peak, the appearance of peaks m/e 180 (M-CH_3OH) and m/e 152 (M-CH_3COOH), together with the presence of 16 protons in its pmr spectrum led to the molecular formula $C_{11}H_{16}O_4$ for this monoacetate (**3**), hence the molecular formula $C_9H_{14}O_3$ for the antifungal constituent (**1**).

C. Chemical structure of the antifungal constituent

On the basis of the foregoing, it was inferred that **1** has a straight chain skeleton

of 8 carbons, which involves a primary hydroxyl group and a carbomethoxyl group at both ends, and a disubstituted allene. The carbon skeleton was confirmed by lithium aluminum hydride reduction of **2**. The reduction gave a diol (**4**) which was identified as 1,8-octanediol by measuring the mixed melting point and its ir spectrum (Fig. IV-5). Thus, four possible structures (**1a**~**1d**) shown in Fig. IV-6 should now be given which differ each other only in the position of allene.

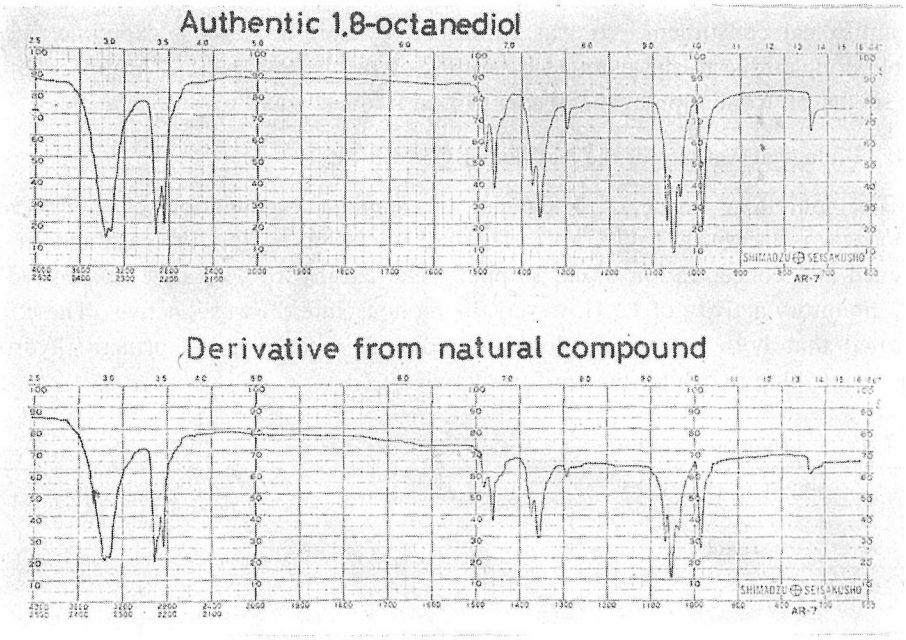
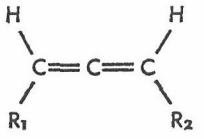


Fig. IV–5. The ir spectra of 1,8-octanediol and **4** (KBr pellet).



1a: $R_1=CH_2OH$ $R_2=(CH_2)_3COOCH_3$

1b: $R_1=CH_2CH_2OH$ $R_2=(CH_2)_2COOCH_3$

1c: $R_1=(CH_2)_2CH_2OH$ $R_2=CH_2COOCH_3$

1d: $R_1=(CH_2)_3CH_2OH$ $R_2=COOCH_3$

Fig. IV–6. Possible structures of **1**.

The structure of **1** was determined by detailed investigations of the pmr spectrum of **1**. A two-proton triplet at 2.38 ppm of **1** was assigned to the methylene protons adjacent to the ester carbonyl group. Then the possible structure (**1d**) should be ruled out. The chemical shift of the double doublet due to the protons attached to the carbon atom bearing the hydroxyl group indicated that hydroxymethyl group is probably located on a double bond. The coupling constants (J=3.2 Hz, 5.8 Hz) of the double doublet was in fair agreement with those observed in the compound (**5**) which is illustrated in Fig. IV-7.[60]

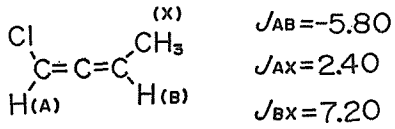


Fig. IV-7. Long-range coupling of **5**.

Thus the structure **1a**, methyl 8-hydroxy-5,6-octadienoate, is most probable for the antifungal constituent. It was confirmed by spin-decoupling experiments: the double doublet due to the methylene protons of the hydroxymethyl group collapsed to a singlet on irradiation at the allenic proton frequency as shown in Fig. IV-3.

IV-4. Biological Activity of the Antifungal Constituent

The inhibitory effect of **1** against the conidia germination of *Cochliobolus miyabeanus* is shown in Table IV-1. The half inhibition against the germination was obtained at a concentration of ca. 10 ppm. The tetrahydro derivative (**2**) showed ca. 30% inhibitory activity of **1**. However, the monoacetate (**3**) was inactive. These facts indicated that both the allenic double bond and, especially, the primary hydroxyl group contribute to exhibit the antifungal activity.

Table IV-1. The Inhibitory Effect of **1** Against Conidia Germination of *Cochliobolus miyabeanus*.

concentration γg/ml	germination ratio %	relative germination %
100	12.9	14.2
50	20.6	22.6
25	20.8	22.8
12.5	38.0	41.7
6.25	69.6	76.4
3.125	78.5	87.3
0 (blank)	91.1	100.0

Table IV-2. Antimicrobial Spectrum of **1**

Saccharomyces cerevisiae	+	*Serratia marcescens*	±
Pichia polymorpha	+	*Bacillus subtilis*	+
Torulopsis famata	±	*Micrococcus lysodeikticus*	±
Candida utilis	−	*Staphylococcus aureus*	−
Rhodotorula glutinis var. rebescens	±	*Sarcina lutea*	−
Streptococcus faecalis	++	*Pseudomonas aeruginosa*	++
Lactobacillus acidophilus	±	*Mucor javanicus Wehmer*	−
Lactobacillus burgaricus	−	*Aspergillus niger van Tiegham*	−
Escherichia coli K 12	+	*Aspergillus oryzae (Ahlb.) Cohn*	−
Aerobacter aerogenes	±	*Penicillium chrysogenun Thom*	±
Proteus vulgaris	+		

The test was performed with paper disk method, and the concentration of sample was 3 mg/ml. ++indicates strong inhibition; +, inhibition; ±, poor inhibition; −, no inhibition.

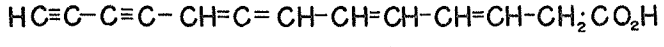
The antimicrobial spectrum with 21 kinds of microorganisms is shown in Table

IV-2. The compound (**1**) showed activitity against *Streptococcus faecalis* and *Pseudomonas aeruginosa*. It is remarkable that **1** shows certain inhibitory activity against *Cochliobolus miyabeanus* while no activity against the 4 kinds of fungi.

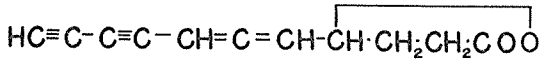
It is very interesting that the compound, which is structurally simple and unique, exhibits the antimicrobial activity. Some additional biological activities may be expected.

IV-5. Naturally Occurring Allenes[61]

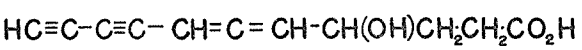
Mycomycin (**6**) was the first naturally occurring allene.[62] It is a fungal metabolite produced by the Actinomycete, *Nocardia acidophilus*. Since then several naturally occurring allenes have been obtained from fungal metabolites. The Basidiomycete fungi have been found to contain nearly 20 allenic metabolites, all of which contain the characteristic diyne-allene grouping[61] and some of them have been reported to show antifungal activity. For example, nemotin (**7**) and nemotinic acid (**8**) were isolated from cultures of an unidentified Basidiomycete and also from *Poria corticola* and *Poria tenuis*.[63] Bu'Lock recently suggested that the marked structural similarity of allenes derived from fungi may be due to their biosynthesis from C_{12} and C_{14} acids produced by the organisms from crepenynic acid by dehydrogenation of the chain and β oxidation.[64]



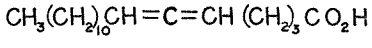
mycomycin (6)



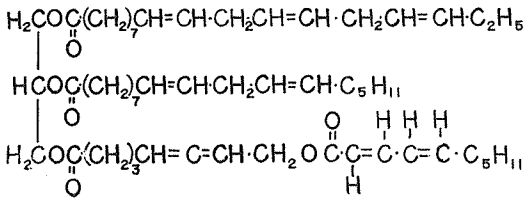
nemotin (7)



nemotinic acid (8)



labellenic acid (10)



(11)

On the other hand, occurrence of allene in higher organisms was proved first by R. Bonnett *et al.*[65] They have isolated fucoxanthin (**9**), a carotenoid pigment, from *Fucus vesiculosus*. Subsequently labellenic acid (**10**) have been isolated from the seed oil of *Leonotis nepetaefolia*.[66] Interestingly an allenic tetraester triglyceride (**11**) containing **1** as a part of its molecule has been isolated from the seed oil of the Chinese tallow tree, *Sapium sebiferum*, a plant of the same genus as *S. japonicum*.[67] The compound **11** is then, expected to occur in *S. japonicum*. Although only a few examples of allenic compounds have been isolated from higher plants, a number of the analogous compounds will be discovered in the near future.

IV-6. Experimental

Proton magnetic resonance (pmr) spectra were recorded in $CDCl_3$ on a Varian A–60 spectrometer. Chemical shifts in the pmr spectra are expressed in ppm from tetramethylsilane as an internal standard and coupling constants in Hz. Singlet, doublet, triplet, double doublet and multiplet are abbreviated to s., d., t., dd. and m., respectively. Infrared spectra were recorded on a Shimadzu AR 275 spectrophotometer and calibrated with 2924, 1603 and 1028 cm^{-1} bands of polystyrene. Optical rotations were measured with a Yanagimoto photomagnetic direct reading polarimeter Model OR–20. Ultraviolet spectra were determined in 95% ethanol with a Hitachi EPS–3T spectrophotometer, mass spectrum with a Hitachi RMU–6D mass Spectrometer. Melting points were determined on a hot stage, and are uncorrected. The following chromatographic materials were used: silicic acid (Mallinckrodt, 100 mesh, U.S.A.), Florisil (Floridin Company, 100~200 mesh), silica gel G (Merck, for thin layer chromatography), Celite 545 washed successively with distilled water and acetone, and then dried at 100°C for 5 hr before use.

Extraction and isolation of the antifungal constituent (*1*)

The fresh leaves (4.0 kg) of *Sapium japonicum* were extracted with methanol (20 liter) at room temperature. The extract was concentrated *in vacuo*, and the concentrate was extracted with ethyl acetate. The ethyl acetate soluble fraction was dried over anhydrous sodium sulfate and evaporated to yield a dark green residue (130 g). A part (22 g) of the residue was adsorbed on silicic acid (75 g) and placed on the top of a column of silicic acid (150 g)-Celite 545 (300 g). The column was eluted in 1.5 liter fractions of benzene containing increasing amounts of ethyl acetate in 10% steps. This process was repeated six times. The activity was found in the fraction eluted with 50% ethyl acetate. All of these fractions were combined to yield a yellow oil (4.3 g), which was purified by column chromatography on Florisil (430 g) eluted in 0.9 liter fractions of the above mentioned solvent in 10% steps. The activity was found in the fraction eluted with 30% ethyl acetate. This fraction (950 mg) was chromatographed on silicic acid (19 g) and eluted with benzene containing 5~10% of ethyl acetate. The active fraction was further purified by preparative thin layer chromatography on silica gel G and 94 mg of the antifungal constituent (**1**) was isolated as an colorless oil in a total yield of 2.4×10^{-3}% of the fresh leaves.

$[\alpha]_D^{12}$ −51.3° (*c* 0.94, $CHCl_3$);

UV λ_{max} nm (ε): 219 (1200), 244 (710, sh.);

IR ν_{max}^{Liquid} cm^{-1}: 3400, 2950, 1965, 1730, 1440, 1365, 1230, 1160, 1015;
PMR: 1.6~2.2 (4H, m.), 2.38 (2H, t. J=6), 3.68 (3H, s.), 4.11 (2H, dd., J=5.8, J=3.2), 5.1~5.5 (2H, m.).

Catalytic hydrogenation of ***1***

The active constituent (**1**) (16 mg) in ethyl acetate (5 ml) was hydrogenated over Adams catalyst until no more hydrogen was absorbed. The catalyst was filtered off and the residue was purified by preparative thin layer chromatography on silica gel G (benzene-ethyl acetate, 1:1) to give a tetrahydro derivative (**2**) (9 mg).
$[\alpha]_D^{22}$ 0° (c 0.90, $CHCl_3$);
UV λ_{max} nm (ε): 210 (end absorption, 226);
IR ν_{max}^{Liquid} cm^{-1}: 3400, 2930, 2850, 1740, 1170;
PMR: 1.2~2.0 (10H, br.), 2.1~2.6 (2H+1H), 3.68 (3H, s.), 3.4~3.8 (2H).

Acetylation of ***1***

The active constituent (**1**) (22 mg) in pyridine (2 ml) and acetic anhydride (4 ml) was allowed to stand overnight at room temperature. The reaction mixture was poured into ice-water, and extracted with ethyl acetate. The extract was washed several times with water, then dried over anhydrous sodium sulfate. The solvent was removed from the extract and the residue was purified by preparative thin layer chromatography on silica gel G (benzene-ethyl acetate, 4:1) to give a monoacetate (**3**) (15 mg) as a colorless oil.
UV λ_{max} nm (ε): 210 (end absorption, 750), 220 (630, sh.);
IR ν_{max}^{Liquid} cm^{-1}: 2950, 1965, 1735, 1450, 1380, 1169, 1015;
PMR: 1.6~2.2 (4H, m.), 2.06 (3H, s.), 2.37 (2H, t, J=6), 3.68 (3H, s.), 4.56 (2H, dd.), 5.1~5.5 (2H, m.);
MS, m/e (relative intensity):
180 (0.17), 170 (8.5), 152 (3.7), 138 (1.2), 110 (9.1), 96 (20), 83 (20), 79 (13), 77 (12), 74 (10), 65 (8.5), 59 (10), 55 (23), 43 (100).

Lithium aluminum hydride reduction of ***2***

The antifungal constituent (**1**) (5 mg) was hydrogenated as described above and the catalyst was filtered off and the filtrate concentrated *in vacuo*. The residue in dry ether (2 ml) was added dropwise under 0°C to a stirred suspension of $LiAlH_4$ (10 mg) in ether (2 ml). After stirring at 0°C for 2 hr, a small quantity of water was added to the reaction mixture, and extracted with ether. The extract was dried over anhydrous sodium sulfate. Removal of the ether gave a crystalline matter (4.6 mg), which was recrystallized from ethyl acetate to give a 1,8-octanediol (**4**) as colorless plates, mp 59~60°C.
IR ν_{max}^{KBr} cm^{-1}: 3380, 3330, 1460, 1380.

Cochliobolus miyabeanus conidia germination test

The conidias of *Cochliobolus miyabeanus* cultivated on Czapek's medium at 28°C for 10~14 days were used. The test was performed according to the procedure reported by Asuyama *et al.*

Antimicrobial spectrum

Twenty one microorganisms listed in Table IV-2 were used for the test. One loopful of a microorganism was cultivated in Bennett's medium (10 ml) at 30°C for 14 hr,

The culture solution (2 ml) was poured into agar medium (10 ml), cooled to 50°C in a test tube, and the test tube was shaken sufficiently. The solution (5 ml) was poured on the petri dish containing agar medium (20 ml) and joggled to overspread flatly. After the medium solidified, the air dried paper disks (7 mm in diameter) which had been dipped in the methanol solution of **1** (3 mg/1 ml) were placed on the surface of the inoculated medium. The antimicrobial activity of **1** was indicated by the zone of inhibition which appeared around the disk after incubation at 30°C for 24 hr.

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